A STUDY OF THE MECHANISM OF PHOSPHATE TRANSPORT IN SEA URCHIN EGGS BY ION EXCHANGE ANALYSIS OF RAPIDLY LABELED COMPOUNDS[1]

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The transport of phosphate into fertilized sea urchin eggs from sea water shows numerous characteristics of an enzymatically controlled transport, in which the mechanism is located in the cell surface. The reaction shows specificity for phosphate; the dependency of rate on phosphate concentration follows Michaelis-Menten kinetics; the reaction is competitively inhibited by arsenate and depends on surface-located sulfhydryl groups. It has a Q_{10} of 2.3, and shows a sharp pH optimum. The transport is unidirectional, carrying phosphate only inward. The transport itself shows little direct dependence on energy metabolism of the eggs. The mechanism is established as a consequence of fertilization (Abelson, 1947; Brooks and Chambers, 1948, 1954; Whiteley, 1949; Chambers and White, 1949, 1954; Lindberg, 1950; Villee and Villee, 1952; Chambers and Mende, 1953; Litchfield and Whiteley, 1959; Whiteley and Chambers, 1960, unpublished data).

To elucidate the mechanism and the process of its differentiation, the reaction catalyzed during the transport needs to be determined. The evidence available concerning the properties of the transport (Whiteley and Chambers, 1960, unpublished data) is compatible with the view that orthophosphate enters by phosphorolysis, perhaps as glucose-1-phosphate formed at the membrane. Lindberg (1950) reported that phosphate is incorporated into adenosine triphosphate at the egg surface. In red cells, it is suggested that phosphate is esterified as 1,3-diphosphoglycerate at the cell surface (Prankerd, 1956) or may be carried through the membrane as ATP[2] (Jonas and Gourley, 1954). Alternatively, a "carrier" leading to the transport of phosphate across the membrane in the form of unchanged orthophosphate can be envisaged, perhaps involving a permease mechanism as proposed for other transports by Rickenberg *et al.* (1956) for bacteria.

Information concerning the nature of the phosphate compound formed in the eggs during the transport would be helpful in clarifying this question. In an attempt to approach this goal, we have determined some of the chemical forms of phosphate that become labeled in fertilized sea urchin eggs during short exposures to radioactive orthophosphate. To increase the accumulation of the first formed compound, the metabolism of the eggs was inhibited by cyanide, azide or monoiodoacetate.

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[2] The following abbreviations have been used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; MIA, monoiodoacetate.

Methods and Materials

The basic method used in all experiments involved the preparation of an acid-soluble extract of normal or inhibited fertilized sea urchin eggs after their exposure to radioactive orthophosphate, followed by the adsorption of phosphates on ion exchange columns and their differential elution and identification by the solvent system of Khym and Cohn (1953) and by paper chromatography. The radioactivity of the eluate was measured continuously with a ratemeter and the activity was recorded graphically.

Labeling of eggs with P^{32}. From 2.0 to 2.5 ml. of gently packed fertilized eggs, washed free of excess sperm, were cultured with gentle stirring at 13.0°–13.5° C. in 200–300 ml. of sea water at pH 8.0 for 100 minutes. At this time, when the phosphate uptake had reached its maximum rate (Whiteley and Chambers, 1960), the eggs were washed with fresh sea water, the volume reduced to 4.5–5.0 ml. and 50 or 100 μc. of P^{32} as orthophosphate were added. The eggs were rapidly agitated by swirling in 40-ml. conical centrifuge tubes at 13.5° C. for a predetermined period, after which they were diluted to 40 ml. with −2° C. sea water in order to stop the uptake and metabolism of phosphate as completely as possible. The eggs were collected by centrifugation and washed 4–5 times with sea water at −2° C., after which 3.0 ml. of iced 0.4 *M* perchloric acid were added. The washing at −2° C. required 12–15 minutes. The final wash had negligible radioactivity.

In experiments with metabolic inhibitors, the cultured eggs were placed in 40 ml. of 5×10^{-3} *M* sodium azide, or 10^{-4} *M* potassium cyanide in sea water at pH 8.0, 13–15 minutes before adding P^{32} to give time for penetration of the inhibitor. Eggs were pretreated with 0.1 *M* sodium monoiodoacetate for 30 minutes. Control eggs of the same culture were left in sea water. The inhibitor was also added in the same concentration to the −2° C. sea water in which the eggs were washed after labeling.

Preparation of acid-soluble extracts. The eggs, in 0.4 *M* perchloric acid, were homogenized, and the homogenates centrifuged 15 minutes at 20,000 *g*. The supernatant extracts were immediately neutralized to pH 7.0 with potassium hydroxide, the perchlorate precipitate removed, and the extract made to a known volume. An aliquot was assayed for total activity, and an appropriate volume used for partition of phosphate compounds by ion exchange chromatography. When a balance sheet of the activity in the acid-soluble and acid-insoluble fractions was desired, the precipitates were washed, and the centrifuged washes neutralized and added to the extract. All operations were performed near 0° C.

Ion exchange chromatography. A slight modification of the method described by Benson (1957) was used. Cations were replaced by hydrogen ions by passing the extract through a Dowex-50 cation exchange column, which was then washed with about 150 ml. of water until the wash was essentially free of radioactivity. The extract and wash were brought to pH 8.0 with ammonium hydroxide and put on a Dowex-1 anion exchange column. Seven eluting solutions (Table I) were used in place of the 9 in the original method, as preliminary experiments showed that very little of the activity was eluted by the two solutions omitted. Elution was at the rate of about 3 ml. per minute. Solutions and columns were maintained at 0° C.

Continuous recording of radioactivity of the eluate from the column. To obtain

a direct graphical record of the chromatogram, the eluate was passed from the column through a small Plexiglas chamber (diameter 25 mm.; depth 3 mm.). The chamber had a thin window made of a piece of cleared 35 mm. Kodak film which was placed in contact with the end window of a Geiger-Müller tube. The radioactivity in the chamber was measured continuously with a rate-meter and recorded graphically by means of a strip chart recorder. Figure 1 is an example of a chromatogram obtained by this procedure. The different fractions were collected in flasks at 0° C.

Radioactivity measurements of the fractions. The total activities of the homogenates, the acid-soluble extracts, and the fractions collected from the columns were assayed on an aliquot of each by means of an end window Geiger-Müller scaler, counting the main samples to a minimum of 5000 counts.

Paper chromatography. While the phosphate compounds of each fraction from the Dowex-1 column can be in part identified from the data on authentic compounds supplied by Benson (1957), further identification of an unknown was made by cochromatography on paper with one or more known compounds of similar anion exchange characteristics. Where there was a very sharp peak of high activity, as in IX in Figure 1, a sample from the top of the peak was chromatographed directly. In other cases, the fraction was first lyophilized. The samples were treated with cation exchange resin to remove salts before application to the paper.

Descending paper chromatograms were run at room temperature using washed (1 N HCl, 2 N oxalic acid, distilled water) Whatman No. 1 paper, and the following solvent systems: (1) methanol:ammonium hydroxide:water (6:1:3) for two hours; (2) isobutyric acid:0.5 *M* ammonium hydroxide (10:6) for 12 hours. Phosphate compounds were located by spraying by the method of Hanes and Isherwood as modified by Bandurski and Axelrod (1951). Adenosine phosphates were also revealed by quenching of ultraviolet fluorescence.

Simplified scanning procedure for radioactive chromatograms. The position of a labeled unknown was determined by attaching the dried chromatogram by masking tape near the right edge of the chart of a Minneapolis-Honeywell Electronik strip chart recorder. The chart, geared to move 20 inches per hour, pulled the chromatogram in front of an end window Geiger-Müller tube clamped in position against the face plate of the chart. The tube was fitted with a brass cap provided with a slit 25 mm. long and 1.6 mm. or 6.6 mm. wide, depending on the level of radioactivity and the resolution needed. The Geiger-Müller tube fed a rate-meter, whose data were recorded dirctly on the strip chart beside the chromatogram. The tracings are in perfect register with the chromatogram, the procedure is simple, and no special equipment is needed. The tracings can be made quantitative by determining the area under the curve.

Ion exchange columns. Dowex-50 hydrogen (California Biochemical Corp., AG 50W-X8, 200–400 mesh) and Dowex-1 chloride (California Biochemical Corp., AG 1-X8, 200–400 mesh) resins were used in columns measuring 9.0 mm. × 100 mm. Both resins were freed of fines before using.

Arginine determinations were made by the method of Dubnoff (1957) on neutral or alkaline solutions containing 5–100 μg./ml. of arginine.

Living materials. Fertilized eggs of the sea urchin *Strongylocentrotus pur-*

puratus were used. The experiments were carried out from November to mid-July. For each experiment, gametes of a single female and male were used. Spawning was induced by electric shocks of 60 volts A. C. or by injection of 4.25% KCl. In the experiments to be reported, 95–100% of the eggs were fertilized, and development of controls through early pluteus larval stages was normal.

Chemicals. P^{32} as orthophosphate was obtained from Oak Ridge, Cat. No. P-1, and used as supplied. Sodium azide and sodium monoiodoacetate were obtained from Eastman Kodak Co.

Results

Distribution of P^{32} in control eggs

1. *Total uptake and partition between acid-soluble and acid-insoluble fractions.* An appreciable amount of the added P^{32} was incorporated by the eggs during brief pulses. For control experiments this ranged from 6.4% to 39.1% during exposures of 10 seconds to 10 minutes. The experiments were not designed to obtain these

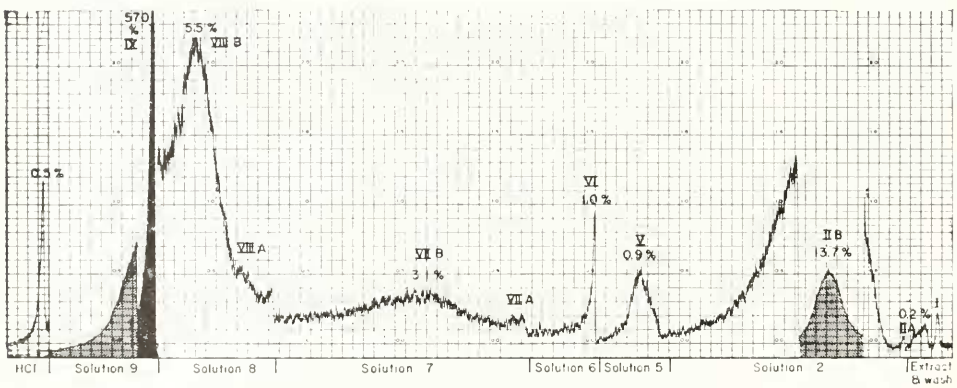

FIGURE 1. Graphical record of the radioactivity of the eluate from a Dowex-1 anion exchange column on which had been adsorbed the acid-soluble extract from control eggs exposed to P^{32} for one minute. The compositions of the eluting solutions, shown at the bottom, are given in Table I. The percentage of the acid-soluble activity is indicated above each peak. Full scale deflection equals 1000 c.p.m. (not hatched); 10,000 c.p.m. (hatched); or 25,000 c.p.m. (solid black).

uptake data with maximal accuracy, and the values are probably less than the maximum possible.

In a few of the control experiments, partition of P^{32} between acid-soluble and acid-insoluble fractions was estimated by assaying the washed acid-insoluble residues in addition to the acid-soluble extract. Most of the activity recovered was in the acid-soluble fraction: 98.4% with a one-minute exposure to P^{32}, and 94.5% with a 10-minute exposure.

2. *Acid-soluble compounds bound by Dowex-50 cation exchange column.* When the neutralized extract was passed through a Dowex-50 column to remove cations, some of the radioactivity became bound at the top of the column and could not be removed by washing with water, although there was a continuous slight leakage of

activity, suggesting the presence of a labile compound. The active compound or its hydrolysis products could be eluted with 1.0 N HCl or 1.0 N NaOH. It is considered to be arginine phosphate on the basis of the following tests. Arginine phosphate is 98% hydrolyzed in 180 minutes at 37° C. in 0.01 N HCl (Mende and Chambers, 1953). A Dowex-50 column containing the unknown material was treated with two bed volumes of 0.01 N HCl, plugged and incubated at 37° C. for 180 minutes. After this, 87% of the activity, presumably due to inorganic phosphate released by hydrolysis, could be washed out of the column with water.

TABLE I

The distribution of P^{32} among the acid-soluble fractions from fertilized sea urchin eggs exposed to P^{32} for one minute. The fractions I–IX are shown graphically in Figure 1 and were eluted from an anion exchange column using the solutions 1–10 shown at the left of the table.

Eluting solution	Vol. ml.	Compounds eluted (Benson, 1957)	Fraction collected	% of total acid-sol. P^{32} activity	Radioactive compound identified
(1) Extract + H_2O wash of Dowex-50 column	150	—	I	0.2	—
(2) 0.025 *M* NH_4Cl + 0.01 *M* $Na_2B_4O_7$	1200	Glucose-1-P Pi	IIA IIB	trace 13.7	Pi Pi
(5) 0.03 *M* NH_4Cl	350	Glucose-6-P Fructose-6-P Ribose-5-P	V	0.9	Glucose-6-P
(6) 0.005 *M* HCl	360	AMP	VI	1.0	Unknown, not AMP
(7) 0.01 *M* HCl	1200	2-P-glycerate ADP	VIIA VIIB	trace 3.1	— ADP
(8) 0.02 *M* HCl + 0.02 *M* KCl	450	Fructose-di-P	VIIIA VIIIB	trace 5.5	— Unknown, not fructose-di-P
(9) 0.02 *M* HCl + 0.2 *M* KCl	450	ATP	IX	57.0	ATP
(10) 1.0 N HCl	150	—	X	0.5	—
		Total from anion column		81.9%	
		Arginine-P from cation column		19.1%	
		Total recovery		101.0%	

Repetition of the hydrolysis removed a further 11% of the activity. That only 87% was hydrolyzed in place of the expected 98% may be due to a lower effective acid concentration in our test. In any case, the presence of a very acid-labile compound was established. In addition, P^{32} and chemically determined arginine (Dubnoff, 1957) were eluted from a Dowex-50 column by 1.0 N NaOH in the same fractions and in roughly proportionate amounts. Lastly, arginine hydrochloride was adsorbed and eluted from Dowex-50 under the same conditions as was the labeled material.

In control experiments with periods of labeling of 10 seconds to 10 minutes, 9% to 27% of the P^{32} was incorporated into arginine phosphate.

3. *Acid-soluble compounds bound by Dowex-1 anion exchange column.* Figure 1 shows a typical record obtained from eluting a Dowex-1 anion exchange column on which had been adsorbed the labeled extract from control eggs exposed to P^{32} for one minute. The eluting solutions are shown in Table I, which also gives the amount of activity present in each fraction, expressed as a percentage of the total activity of the acid-soluble fraction, and summarizes the identifications of the fractions within the limitations of the observations so far made.

In Figure 1, peak I represents material, usually less than 0.5% of the total, that was not bound to the column due to overloading or too rapid application.

The two compounds known to be eluted by solution 2 are glucose-1-phosphate and inorganic phosphate. Peak IIB, which has 13.7% of the activity, is due to inorganic phosphate. Its elution from the column began after 50–100 ml. of solution 2 had passed through. Labeled inorganic phosphate, added by itself, is eluted similarly. In addition, when cochromatographed on paper with inorganic phosphate, using the two solvent systems described in methods, the radioactivity of the eluate and the added orthophosphate coincided. However, although strongly radioactive, there was insufficient inorganic phosphate present in the paper chromatogram to give any phosphate reaction when sprayed; the specific activity of the inorganic phosphate must be very high. Peak IIA, which was very small in this experiment, appeared a little earlier than the expected glucose-1-phosphate. The fraction was lyophilized and cochromatographed on paper with glucose-1-phosphate and inorganic phosphate. The small amount of radioactivity in the fraction coincided with inorganic phosphate, and not with glucose-1-phosphate. The latter was present in the extracts and gave a phosphate spray reaction on the paper, but had little or no label. A compound coinciding with glucose-1-phosphate was also present in the paper chromatogram of peak IIB. It was unlabeled.

Peak V is usually small, in this experiment comprising only 0.9% of the total activity. The compounds which are known to be eluted by solvent 5 are glucose-6-phosphate, fructose-6-phosphate, and ribose-5-phosphate. Cochromatography on paper of the lyophilized eluate showed that the radioactivity was not due to residual inorganic phosphate nor to fructose-6-phosphate. It coincided with glucose-6-phosphate.

Peak VI, which had 1.0% of the activity, is due to a substance with the elution characteristics of adenosine-5-phosphate. However, the ultraviolet absorption of the lyophilized fraction was inconclusive. Cochromatography on paper with adenosine-5-phosphate and inorganic phosphate showed that the radioactive compound was neither of these, nor was it residual glucose-6-phosphate from Peak V. From its ion exchange characteristics it should be a monophosphate.

Two substances are usually eluted with solution 7. The first of these, VIIA, is a minor component which might be 2-phosphoglycerate. The second component, with 3.1% of the total activity, has the elution characteristics of adenosine diphosphate and we have confirmed this by cochromatography.

Solution 8 is known to elute fructose-1, 6-diphosphate. In Figure 1 two minor unknown components, pooled in VIIIA, precede the major component, VIIIB, which accounts for 5.5% of the total activity. Paper chromatography of the lyo-

philized fraction VIIIB was difficult due to the high concentration of salts in the concentrated samples, but in no case did the radioactivity coincide with fructose-diphosphate nor is it due to inorganic phosphate hydrolyzed from the ATP still on the column. Though radioactive, the component was not revealed by the phosphate spray reagent, and presumably has a relatively high specific activity. The identity of this major component is therefore unknown, but it should be a diacidic substance.

Solution 9 elutes ATP. In this experiment, 57.0% of the activity was in fraction IX. The activity was due to ATP, as determined both by cochromatography, and by the ultraviolet absorption spectrum. Frequently a small peak appears on the trailing shoulder of the ATP fraction. This could include other nucleoside triphosphates, known to be eluted in this region (Cohn, 1957).

A residual 0.5% of the activity remained on the column. This was eluted with 1.0 N HCl.

Recovery from the columns was usually good. In this experiment, the total recovery was 101.0%, 19.1% being recovered from the Dowex-50 column and 81.9% from the Dowex-1 column.

In Table II data from this and other control experiments in which the eggs were exposed to P^{32} for one or 10 minutes have been tabulated. The partitioning of P^{32} among the fractions described is similar for both of these time intervals.

Effect of metabolic inhibitors on distribution of P^{32}

It has been shown previously (Litchfield and Whiteley, 1959; Whiteley and Chambers, 1960, unpublished data) that 5×10^{-5} *M* azide and 10^{-5} *M* cyanide have no effect upon the uptake of phosphate by the eggs although they have an inhibiting effect upon cleavage. In the present experiments with 5×10^{-3} *M* azide, 10^{-4} *M* cyanide and with 0.1 *M* monoiodoacetate, the extent of incorporation was as much as in the corresponding controls and in some cases more (Table II). Despite this strong uptake, these agents caused considerable changes in the distribution of P^{32} among the acid-soluble compounds after pulses of one or 10 minutes, as shown in Table II. In the presence of azide, Experiments 1 and 2, there is a great decrease in the percentage of label incorporated into the high energy phosphate compounds. In the 10-minute experiment, the ATP was reduced from 55.3% to 23.0%, and in the one-minute experiment, from 53.7% to 14.3%. The drop in arginine phosphate was nearly complete: from 11.9% to 0.3% and from 27.0% to 1.2%, respectively. A corresponding increase appeared in the labeled inorganic phosphate (the sum of IIA and IIB), which rose from 11.7% to 58.7% in the 10-minute P^{32} pulse, and from 7.7% to 76.9% in the one-minute pulse. There is thus a loss of 43.9% from the two high energy compounds and a gain of 47.0% in orthophosphate in the 10-minute experiment, and a 65.2% loss and 69.2% gain of these compounds in the one-minute experiment.

Very similar results were obtained using potassium cyanide as inhibitor. These are shown in Experiment 3 of Table II. Again there was a very great decrease in labeled high energy phosphate (arginine phosphate and ATP) and an equivalent increase in labeled inorganic phosphate. As with azide, the arginine phosphate fraction was essentially unlabeled.

TABLE II

The effect of metabolic inhibitors on the distribution of P^{32} among the acid-soluble fractions from fertilized sea urchin eggs exposed to P^{32}

	Duration of P^{32} pulse, minutes	% of P^{32} taken up by eggs	Eluted from Dowex-50 arginine-P %	Fractions eluted from Dowex-1 column											Recovery %
				I %	IIA %	IIB Pi %	V Glucose-6-P %	VI %	VIIA %	VIIB ADP %	VIIIA %	VIIIB %	IX ATP %	1 N HCl strip %	
Exp. 1. Control	10	39.1	11.9	0.1	0.2	11.5	0.9	1.4	0.3	1.3	0.7	3.3	55.3	0.3	87.2
5×10^{-3} *M* NaN_3	10	47.8	0.3	0.2	0.5	58.2	1.0	1.7	trace	4.3	1.1	1.8	23.0	trace	92.1
Exp. 2. Control	1	6.4	27.0	0.4	0.9	6.8	0.8	0.8	trace	2.0	—	4.2	53.7	0.3	97.0
5×10^{-3} *M* NaN_3	1	9.7	1.2	0	0	76.9	1.9	1.2	0	4.2	—	3.9	14.3	0.2	104.8
Exp. 3. Control	1	29.3	19.1	0.2	trace	13.7	0.9	1.0	trace	3.1	trace	5.5	57.0	0.5	101.0
10^{-4} *M* KCN	1	42.1	0.6	trace	trace	71.7	1.8	1.2	trace	4.0	—	2.6	16.9	0.2	99.4
Exp. 4. Control	1	15.0	9.0	trace	2.3	16.1	5.7	0.4	trace	2.2	1.4	4.7	47.8	0.3	89.8
0.1 *M* MIA	1	14.9	9.0	trace	trace	35.0	3.3	0.5	trace	1.9	1.2	3.4	38.3	trace	92.6
Exp. 5. Control	1	24.5	12.5	0.9	0.8	6.1	5.9	1.3	trace	4.1	1.7	4.7	61.2	0.7	99.9

Changes in the other components are all small, but show the same pattern for both the azide and cyanide experiments. These include a moderate increase in label in glucose-6-phosphate, a small increase in fraction VI, a somewhat larger increase in ADP, and an appreciable drop in fraction VIII.

Both azide and cyanide caused the first cleavage of the eggs to be delayed by a length of time equal to or longer than the time of exposure. These inhibitions were largely reversible.

Less clear-cut changes in the distribution of P^{32} were obtained in the presence of 0.1 *M* monoiodoacetate (Table II). There was a reduction of 9.5% in ATP (38.3% in the experimental eggs compared with 47.8% in the controls) and an increase of 16.6% in inorganic phosphate (35.0% as compared with 18.4% for the controls). There was no change in the labeling of arginine phosphate. Both glucose-6-phosphate and fraction VIII were less strongly labeled in the presence of monoiodoacetate. A 30-minute exposure to the inhibitor caused a delay in the first cleavage of 14 minutes. On return to sea water subsequent development of these eggs was blocked in the gastrula stage.

Effect of length of P^{32} pulse on the distribution of P^{32} among the acid-soluble compounds

In an effort to establish the time sequence of labeling, the eggs were exposed to P^{32} for 10 minutes and for one minute in the control experiments described in Table II. It is clear that there is no consistent difference in the relative distribution of activity during these intervals. A technical weakness in these experiments was the relatively long time, about 10–15 minutes, required to wash the eggs free of all extracellular P^{32}. Although these washes were carried out in sea water at −2° C., undoubtedly some metabolism of phosphate continued during the washing period. In order to minimize this and so determine which phosphate compounds are labeled initially, experiments with 30-second and 10-second exposures to P^{32} were carried out with a modified procedure. At the end of these brief periods, the 5-ml. egg suspensions were diluted to 40.0 ml. with sea water at −2° C., centrifuged for 30 seconds at 500 *g* in the cold, the supernatant carefully removed, and perchloric acid added to the eggs without further washing. In this way, the time from the end of the P^{32} pulse to the addition of perchloric acid was reduced to two minutes at about 0° C. The supernatant sea water removed was assayed for P^{32} activity. The extracts were fractionated on the ion exchange columns and corrections were made on the assumption that 10% to 25% of the packed egg volume was due to extracellular fluid. The results of these experiments are in Table III, calculated on the basis that either 10% or 25% of the activity was due to extracellular radio-orthophosphate. The true value can be expected to lie between these extremes. The results are preliminary but are believed to approach the initial values more closely than do those from the one-minute and 10-minute experiments. They show again that more than half of the P^{32} is converted to high energy phosphates, even in these brief intervals, though the percentages are less than for the one- or 10-minute experiments. Further, there is a decided increase in the percentage of label in the orthophosphate fraction in the short-term experiments, reaching a value that may be as high as 39% in the 10-second experiment.

TABLE III

The distribution of P^{32} among the acid-soluble fractions from fertilized sea urchin eggs exposed to very short P^{32} pulses. Corrections have been made for P^{32} trapped in the intercellular spaces.

	Duration of P^{32} pulse, seconds	% of P^{32} taken up by eggs	Eluted from Dowex-50 arginine-P %	Fractions eluted from Dowex-1 column								Recovery %
				I %	IIA+B Pi %	V Glucose-6-P %	VI %	VIIA+B ADP %	VIIIA+B %	IX ATP %	1 N HCl strip %	
Exp. 6.												
Corrected for 10% intercellular space	10	18.4	12.0	1.3	39.2	0.3	0.6	1.0	2.7	39.7	0.2	97.0
Corrected for 25% intercellular space	10	17.1	14.9	1.6	24.8	0.4	0.7	1.2	3.3	49.0	0.3	96.2
Exp. 7.												
Corrected for 10% intercellular space	30	21.8	15.5	3.5	31.4	0.7	0.5	1.6	2.6	41.2	—	96.9
Corrected for 25% intercellular space	30	20.5	17.8	4.0	20.8	0.7	0.5	1.8	3.0	47.7	—	96.3

Discussion

Three different hypotheses have been advanced to explain the transport of substances across membranes where it can be shown that simple diffusion is not an adequate explanation. The first involves specific sites in the membrane where substances are either adsorbed to, or chemically combined with, a carrier molecule. The substance is liberated to the cytoplasm chemically unchanged. The second concept envisages the chemical change of the molecule at the cell surface during the transport, liberating it in a new chemical form to the cytoplasm. The third is that of pinocytosis. In the absence of evidence for pinocytosis in sea urchin eggs, this mechanism need not be considered here.

The present data may be considered with respect to the first two mechanisms. Identification of the first labeled form of phosphate, the transport form, would settle the question. The present biological material offers certain simplifications over other cells where phosphate transport has been studied: yeast (Rothstein, 1955), erythrocytes (Gourley, 1952; Prankerd, 1956), bacteria (Mitchell, 1959) and liver cells (Sacks, 1951). In sea urchin eggs the transport is strictly unidirectional and results in a very pronounced accumulation; it is not inhibited by cyanide, azide, monoiodoacetate, anaerobiosis, and 2,4-dinitrophenol (Litchfield and Whiteley, 1959; Whiteley and Chambers, 1960, unpublished data) nor does it require exogenous nutrients.

In other instances, precursor relationships among labeled phosphate compounds have been sought by determining relative specific activities, in order to establish the sequence in which phosphate passes through the membranes and into the metabolic pools. In the present study, the transport form of phosphate has been sought by taking advantage of the fact that the inhibitors do not block uptake, but do interfere with metabolic flow of phosphate. Thus, if an inhibitor does not inhibit the uptake but causes a great increase in label in one component and a decrease in others, then the latter compounds are unlikely candidates for the transport form, while the former compound is likely.

Lindberg (1950) proposed that phosphate penetration in sea urchin eggs was by formation of ATP near the surface. This mechanism was rendered improbable by the observations, cited above, that inhibitors of energy metabolism do not depress the rate of uptake of phosphate. The present observations substantiate that more than 50% of the radioactive phosphate is metabolized to ATP and arginine phosphate, even in 10-second pulses, and in the one- and 10-minute experiments the value is about 70%. But the fact that the inhibitors tested greatly depress the entrance of P^{32} into the high energy phosphates and increase the percentage in the inorganic phosphate pool is a strong argument against any mechanism involving the initial formation of any high energy phosphate compound.

The properties of the transport mechanism previously determined would permit the hypothesis that transport is by means of a surface-located glycogen phosphorylase which would phosphorylyze glycogen from the inside with phosphate from the outside, liberating glucose-1-phosphate into the cell. Such a mechanism has also been indicated as possible in phosphate transport in liver cells (Sacks, 1951). In this event, a high percentage of labeling of glucose-1-phosphate would be expected, but this was not found. In addition, the observed increase in percentage of P^{32} as inorganic phosphate and decrease in high energy forms in the presence of the

inhibitors would not be expected. It is concluded that phosphate does not enter as glucose-1-phosphate.

In erythrocytes, Prankerd and Altman (1954) suggested that phosphate enters in the reaction, forming 1,3-diphosphoglycerate. In the sea urchin eggs, such a mechanism would account for the lack of label in glucose-1-phosphate and could account for the observations that azide increased the label in orthophosphate at the expense of ATP and arginine phosphate. According to Spiegelman *et al.* (1948), azide uncouples the oxidation of triosephosphate from the generation of ATP from 1,3-diphosphoglycerate. Labeled phosphate picked up at the membrane would thus be released in glycolysis as orthophosphate rather than as high energy phosphate. However, this explanation would not account for the similar results with cyanide, which has no such uncoupling effect. Further, monoiodoacetate, which inhibits triose dehydrogenase, would not cause the observed accumulation of label in the inorganic phosphate pool and should depress total uptake, especially if 1,3-diphosphoglycerate formation occurs at the surface. No such depression is observed.

For all these possible mechanisms, or any involving group-transferring reactions (Mitchell, 1959), one would expect the activity present in inorganic phosphate to increase with increased time of exposure to P^{32} as the label is cycled and released into the inorganic pool. On the contrary, the experiments indicate that the proportion of label present as inorganic phosphate to that as high energy phosphate is much higher in the short pulses than in the longer ones. In the 10-second experiment, this proportion is between 0.77 and 0.40 and in the one- and 10-minute experiments, it averages 0.15.

While the evidence of these experiments is opposed to the mechanisms so far described, they and the other known facts of phosphate transport in sea urchin eggs are not incompatible with a carrier molecule type of transport system, which would release inorganic phosphate unchanged into the metabolic pool in the eggs. The great increase in activity of the inorganic phosphate in metabolically blocked cells would be due to a continued high rate of pumping of inorganic radioactive phosphate and its greatly reduced use in oxidative phosphorylation to form ATP and arginine phosphate. The increased proportion of label appearing as inorganic phosphate as the pulse is shortened is in support of the idea that the transport form is orthophosphate. Such a carrier could have the specific properties of an enzyme, as, for example, the permeases of Rickenberg *et al.* (1956). The kinetic, specificity, and inhibition properties described by Whiteley and Chambers (unpublished data), and the highly polarized character of the transport are compatible with this suggestion. The negligible activity found in glucose-1-phosphate suggests that either a high concentration of this substance is present initially in the cell, and little is formed during exposure to P^{32}, or that the cytoplasm has at least two pools of inorganic phosphate, one of which receives labeled phosphate from the outside and supplies it to many metabolic reactions, and a second, unlabeled pool, which donates phosphate for the formation of glucose-1-phosphate. The carrier or permease model does not easily account for the observed lack of direct dependence of the transport on energy metabolism of the egg, particularly since accumulation takes place against a remarkable concentration gradient (Whiteley and Chambers, unpublished data). Conceivably, this transport has a high priority for the available energy mobilized in the cells in the presence of inhibitors.

Summary

1. The mechanism involved in the transport of phosphate into fertilized sea urchin eggs has been studied by exposing eggs to pulses of P^{32} and examining the radioactive compounds formed. These have been identified in part by ion exchange and paper chromatography in control eggs and in the presence of metabolic inhibitors.

2. Control eggs of *Strongylocentrotus purpuratus* at the two-cell stage incorporated 6.4% to 39.1% of the P^{32} from sea water in 10 seconds to 10 minutes; most of this (98.4% for a one-minute pulse, and 94.5% for a 10-minute pulse) was in perchloric acid-soluble compounds.

3. The distribution of the label after one-minute and 10-minute pulses was similar. In a representative one-minute experiment 27.0% was found in arginine phosphate; 7.7% in inorganic phosphate; 0.8% in glucose-6-phosphate; 2.0% in ADP; 53.7% in ATP; and 5.7% was distributed among 5 unidentified fractions. Glucose-1-phosphate, while present in the extract, was without significant label.

4. In experiments in which one-minute and 10-minute pulses of P^{32} were given to eggs in the presence of 5×10^{-3} *M* sodium azide, or 10^{-4} *M* potassium cyanide, the incorporation of label was as much or more than in the controls, but its distribution was greatly altered, with similar changes for both azide and cyanide. In a one-minute pulse in the presence of azide, incorporation into arginine phosphate was reduced to 1.2% and into ATP to 14.3% while inorganic phosphate increased to 76.9%: a decrease of 65.2% in high energy phosphates and an increase of 69.2% in inorganic phosphate. Minor changes occurred in other fractions. Similar, but much less marked changes were produced by 0.1 *M* monoiodoacetate.

5. A modified procedure was used with very short P^{32} pulses of 10 and 30 seconds, in an attempt to determine the initially labeled compound. The shorter the pulse, the greater was the percentage incorporation into inorganic phosphate, with parallel decreases in arginine-phosphate and ATP.

6. The results are considered to be incompatible with any mechanism for transport involving the initial formation of high energy phosphate; or with transport as the result of surface phosphorolysis of glycogen to form glucose-1-phosphate; or with transport resulting from glycolytic esterifications. The results are compatible with a carrier type of transport which would release inorganic phosphate unchanged into the metabolic pool in the eggs.

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